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Adenoviral vectors capable of replication improve the efficacy of HSVtk/GCV suicide gene therapy of cancer

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A major obstacle to the success of gene therapy strategies that directly target cancer cells is the poor vector distribution within solid tumors. To address this problem, we developed an E1b 55 kDa attenuated, replication-competent adenovirus (Ad.TK^{RC}) which expresses the herpes simplex-1 thymidine kinase (HSVtk) gene to sensitize tumors to ganciclovir (GCV). Efficacy of this combined strategy was tested in nude mice with subcutaneous human A375 melanoma and ME180 cervical carcinomas. Intratumoral injection of a replication-defective adenoviral vector expressing HSVtk (Ad.TK) followed by GCV treatment resulted in doubling of the survival time of mice bearing A375 tumors and 20% long-term survival of mice with ME180 tumors. Treatment of tumors with Ad.TK^{RC} without GCV resulted in a similar antitumor effect, confirming that the replicating vector has an oncolytic effect. When GCV was initiated 3 days after Ad.TK^{RC} injection, survival of mice with each tumor type was greatly prolonged, with 60% of animals with ME180 tumors surviving for over 160 days. These results confirm that both the oncolysis caused by a replicating virus and suicide/prodrug gene therapy with HSVtk/GCV have potent antitumor effects. When combined, these two approaches are complementary resulting in a significantly improved treatment outcome.

Keywords: *gene therapy; replication-competent adenovirus; thymidine kinase; ganciclovir; melanoma; cervical cancer*

Introduction

Successful application of suicide gene therapy strategies for cancer is limited by the ability of current replication-deficient viral vectors to transduce the majority of cells within a tumor. The presumed need to genetically modify every tumor cell may be partially overcome by the HSV*tk*/GCV system since it is often associated with a 'bystander effect' which extends toxic effects of the activated prodrug to nearby untransduced wild-type cells.[1,2]

To increase the transduction efficiency, replication-defective viral constructs expressing HSV*tk* have been cotransfected with wild-type virus[3,4] or viral genes crucial for replication[5] in order to allow replication and spread of the vector. Other studies have explored the direct inoculation of xenogenic murine retroviral producer cells into cerebral tumors in an effort to achieve continuous local vector production.[6,7]

Certain attenuated replication-competent viruses have been extensively used in human vaccination programs with few unwanted effects.[8] In addition, over the past 40 years there have been several clinical trials in which replication-competent wild-type viruses, including adenoviruses, were intratumorally administered to patients with various malignancies.[9–11] 'Virotherapy' of cancer was abandoned since only few responses were reported, its effects were unpredictable and the development of more active chemotherapeutic agents supplanted it. In the light of the recent findings of Bischoff and colleagues[12] demonstrating that an E1b 55 kDa attenuated adenovirus can preferentially replicate and lyse p53-dysfunctional tumor cells but not in adjacent normal tissue, this approach is now being re-evaluated.

As an extension of this approach, we and others have developed E1b 55 kDa-deleted adenoviral vectors expressing HSV*tk* or a cytosine deaminase/HSV*tk* fusion gene.[13] In this report we describe a strategy for cancer gene therapy that combines the attributes of a standard replication-deficient adenoviral vector expressing HSV*tk* (Ad.TK), with high suicide gene expression, and an E1b 55 kDa attenuated replication-competent adenovirus, for cytotoxic spread throughout tumors. We compared the efficacy of this approach with that of a traditional replication-deficient Ad.TK in a melanoma and cervical cancer xenograft tumor model.

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Results

Analysis of HSV*tk* expression, replication and E1b 55 kDa of Ad.TK^{RC}

Beginning with a first generation E1- and E3-deleted adenoviral vector, we constructed Ad.TK^{RC} with the human CMV-IE promoter driving the HSV*tk*, Ad5 E1a and E1b 19 kDa genes (Figure 1). To test its HSV*tk* gene expression, we analyzed by Northern blot RNA from A375 cells infected with Ad.TK^{RC}, Ad.TK or Ad5^{WT} (Figure 2a). HSV*tk* transcripts could be readily detected

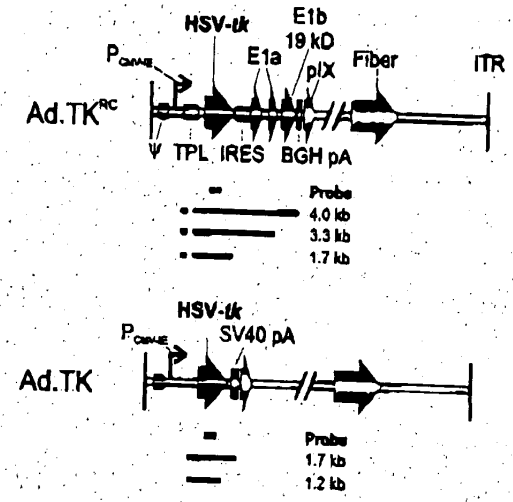

Figure 1 Schematic representation of the HSVtk expressing adenoviruses used in this study. Both viruses are E3 deleted and carry in the E1 region a CMV-IE promoter-driven HSVtk expression cassette which is terminated by the bovine growth hormone (BGH) or SV40 polyadenylation signal (pA), respectively. The cassette is flanked upstream by the Ad5 packaging sequence (ψ) and downstream by Ad5 pIX. In addition, in the replication-competent Ad.TK^RC the HSVtk transcripts have the Ad5 tripartite leader (TPL) sequence and they are coupled via an internal ribosome entry site (IRES) of encephalomyocarditis virus[43] with that of the Ad5 E1a and E1b 19 kDa genes. The HSVtk transcript sizes and the localization of the probe used for Northern blot hybridization (Figure 2) are indicated.

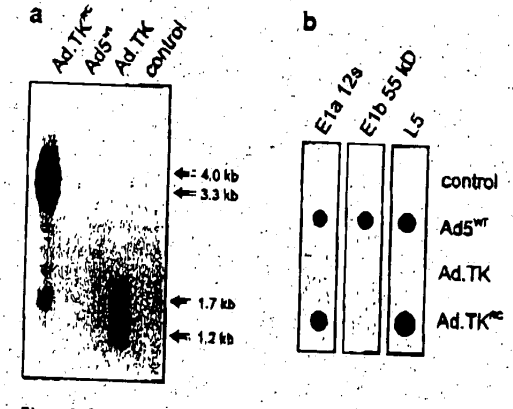

Figure 2 Expression of HSVtk, Ad5 E1 and L5 genes in A375 cells infected with different adenoviruses. (a) Northern analysis of HSVtk expression in A375 cells. Adenoviruses used for infection and molecular sizes are indicated. The hybridization probe and expected HSVtk transcripts are shown in Figure 1. (b) RNA dot blot analysis of Ad5 E1a 12s, E1b 55 kDa and fiber (L5) gene expression in A375 cells infected with the specified adenoviruses.

in Ad.TK^RC and Ad.TK-infected cells but not in the Ad5^WT or uninfected cells. In the Ad.TK^RC-infected cells we observed two predominant HSV*tk* messages which differed in size by about 700 bp. The most likely explanation for this is that the longer message terminates at the BGH pA and the shorter at the viral E1a pA, since viral gene transcription is not terminated between the E1a and the adjacent downstream E1b gene in cells infected with Ad5^WT.[14] Since in Ad.TK^RC-infected cells the transcription of HSV*tk* is transcriptionally coupled with an internal ribosome entry site (IRES) to that of the E1a and E1b 19 kDa genes, the full length HSV*tk* message is about 2.3 kb longer than in cells infected with the replication-deficient Ad.TK. The least abundant transcript represents presumably only the HSV*tk* ORF. In the Ad.TK-infected cells there were two major HSV*tk* transcripts detectable. The approximately 1.7 kb message probably terminated at the SV40 pA site and the shorter at the HSV*tk* pA signals (48 and 61 bp downstream of the *tk* ORF).

To verify that the HSV*tk* expression of Ad.TK^RC is functional, we analyzed extracts of the adenovirus-infected A375 cells by HPLC for GCV metabolites and their incorporation into the genomic DNA. In cells infected with Ad.TK^RC or Ad.TK, high levels of GCV-diphosphate (394 and 244 pmol/10^6 cells, respectively) and GCV-triphosphate (3610 and 1670 pmol/10^6 cells, respectively) were detected. Accordingly, A375 cells infected with Ad.TK^RC and Ad.TK showed a marked incorporation of ^{3}H-GCV into macromolecular DNA (7.41 ± 0.35 and 6.81 ± 0.44 pmol/10^6 cells, respectively). Uninfected control cells or cells infected with Ad5^WT showed negligible levels (<0.1 pmol/10^6 cells) of GCV metabolites and ^{3}H-GCV DNA incorporation.

Dot blot analysis of RNA from A375 cells infected with Ad.TK^RC confirmed the lack of E1b 55 kDa transcripts (Figure 2b). The E1a 12s and L5 mRNA of Ad.TK^RC could be readily detected suggesting viral DNA replication and late protein synthesis.[15,16] As expected, wild-type Ad5-infected cells expressed all three viral RNAs and in the Ad.TK-transduced cells none of the viral RNAs were detectable by RNA dot blot.

To assess whether the E1b 55 kDa-deleted Ad.TK^RC preferentially replicates in p53-dysfunctional cells as described previously,[12] we infected human p53-positive lung carcinoma cells (A549) and p53-mutant cervical cancer cells (C33A) with a series of viruses (Figure 3). As expected, infection with wild-type Ad5 resulted in cytopathic effect and cell lysis regardless of the p53 status of the cells. The replication-defective adenovirus Ad.TK did not result in any detectable cytopathic effect in the cells tested. In contrast, Ad.TK^RC caused cytopathic effect only in the p53-mutant C33A cells but not in the A549 cells.

Transduction efficiency and bystander effect of cervical and melanoma cell lines

Several human cervical cancer and melanoma cell lines were tested *in vitro* for transduction efficiency with a replication-deficient adenovirus expressing green fluorescent protein (Ad.GFP) and their HSV*tk* associated bystander effect. At a multiplicity of infection (MOI) of 10, the median transduction efficiency of the human melanoma cell lines A375, SK-MEL 2 and SK-MEL 31 were 48.2, 99.5 and 31.9%. In contrast, all human cervical cancer cell lines tested (ME180, CaSki, HeLa, SW756 and SiHa) under the same conditions had a transduceability of more than 90%.

After exposure of a mixture of 10% HSV*tk*-positive and 90% wild-type cells to 10 μM GCV for 24 h, ^{3}H-thymidine incorporation was inhibited by 67% (A375), 25% (SK MEL-2), 21% (SK MEL-31), 45% (ME180), 96% (CaSki),

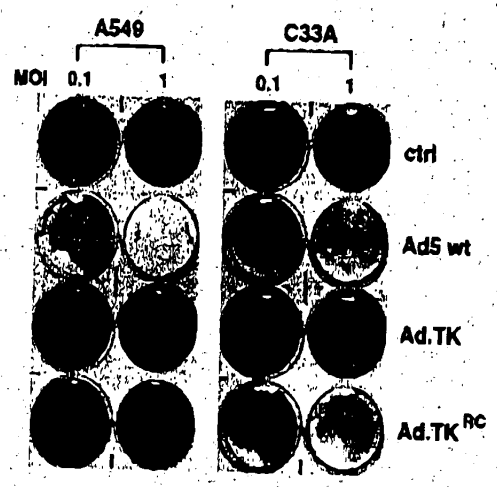

Figure 3 Effects of Ad.TK^{RC}, Ad.TK and wild-type Ad5 on the p53-positive human lung carcinoma cells (A549) and p53-mutant human cervical cancer cells (C33A). The cell monolayers were infected with the viruses at MOIs of 0.1 or 1. Uninfected cells were used as the control. Five days after infection, viable cells were stained with crystal violet.

90% (HeLa), 53% (SW756) and 13% (SiHa) compared with untreated cell mixtures. These data demonstrate that different cell lines derived from similar tumors can vary both in their susceptibility to adenovirus gene transfer and in their HSV*tk*/GCV bystander effect. We chose A375 melanoma and ME180 cervical cancer cells for our *in vivo* studies. A375 cells transduce with Ad5 only half as well as ME180, but had a somewhat higher metabolic bystander effect.

To evaluate the *in situ* transduction efficiency of A375, tumor xenografts growing in nude mice were intratumorally injected 10^9 p.f.u. of the replication-deficient Ad.GFP vector. In five independent experiments, the median percentage of GFP expressing cells found in tumors resected at 60 h was 7.3%, with a range of 3.1–13.5%.

To determine the transduction efficiency needed for GCV-mediated elimination of A375 xenografts, we established tumors consisting of mixtures of different proportions of stable HSV*tk* expressing cells and untransduced A375 cells followed by GCV administration (100 mg/kg twice a day for 7 days). When between 5 and 20% of the implanted tumor cell mixtures expressed HSV*tk*, two of 12 animals became long-term disease-free survivors. The tumors regressed completely in all animals if the xenografts consisted of 100% HSV*tk*-positive A375 cells.

In vivo efficacy of Ad.TK^{RC} compared with Ad.TK

Nude mice bearing subcutaneous A375 melanoma or ME180 cervical carcinoma xenografts were randomly assigned to treatment groups when the tumors reached a volume of 100–150 mm^3. Control tumor-bearing mice were given an intratumoral injection of PBS (100 µl) and then 1 day later a 7-day course of GCV was initiated. Their responses were compared with tumor-bearing groups treated with an intratumoral injection of 10^9 p.f.u. Ad.TK or Ad.TK^{RC} (in 100 µl) followed by GCV 100 mg/kg twice a day for 7 days beginning 24 or 72 h later. Rapidly growing A375 melanoma tumors developed in the control groups that required their euthanization within 27 days (Figure 4a). Mice with A375 tumors treated with Ad.TK alone survived slightly longer than control animals, while the addition of GCV treatment to these animals beginning 1 day after vector injection resulted in a doubling of their survival times. This confirms the antitumor effect of the HSV*tk*/GCV treatment, but the lack of long-term cures also illustrates its limitations in the A375 tumor model. A375 tumor-bearing mice treated with Ad.TK^{RC} followed by GCV treatment beginning 1 day later had a nearly identical pattern of response. If GCV treatment was delayed until 3 days after injection of Ad.TK^{RC} the initial antitumor response was greatly improved although over the course of observation half of the animals relapsed (Table 1). Interestingly, treatment with Ad.TK^{RC} alone resulted in 20% long-term disease-free survival showing efficacy of replication-competent virus alone against this tumor.

A similar treatment regimen was tested in nude mice bearing ME180 cervical carcinoma. Again, response rates to treatment with Ad.TK plus GCV or Ad.TK^{RC} without GCV were similar and resulted in a doubling of median survival times and long-term disease-free survivors of 20–30% (Figure 4b). If GCV treatment was delayed until 3 days after viral inoculation, responses were dramatically

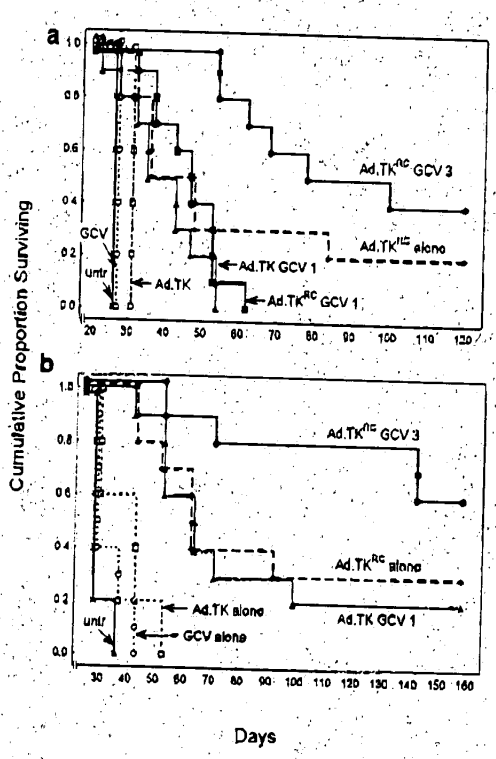

Figure 4 Kaplan–Meier survival analysis of mice with subcutaneous A375 melanoma (a) and ME180 cervical cancer (b) xenografts treated with a traditional replication-deficient Ad.TK or a replication-competent Ad.TK^{RC}. The viruses were administered alone or followed by GCV treatment (100 mg/kg twice a day for 7 days) starting on day 1 (GCV 1) or day 3 (GCV 3) after virus injection.

Table 1 Comparison of animal survival between different treatment groups

Group	A375 xenografts			ME180 xenografts		
	Group size (n)	Long-term survivors (n)	Median survival (d)	Group size (n)	Long-term survivors (n)	Median survival (d)
Untreated	5	0	27	5	0	29
GCV alone	5	0	27	10	0	29
Ad.TK alone	5	0	32	5	0	43
Ad.TK GCV 1	10	0	39.5[a]	10	2	64[b]
Ad.TK^{RC}	10	2	41.5	10	3	64
Ad.TK^{RC} GCV 1	10	0	47	ND	ND	ND
Ad.TK^{RC} GCV 3	10	4[c]	90[a]	10	6	158[b]

[a] $P = 0.00003$.
[b] $P = 0.023$.
[c] Three of the four animals tumor free at day 120; all other long-term survivors were tumor free at day 120 (A375) or 160 (ME180).
d, days; ND, not done.

enhanced with 60% of the animals surviving for over 160 days.

Discussion

We generated a dual mechanism adenoviral gene transfer vector (Ad.TK^{RC}) expressing HSV*tk*, which is capable of replication and spread within certain tumors. In contrast to dl1520,[12,17] Ad.TK^{RC} expresses a suicide gene and the E1a, E1b 19 kDa genes are driven by the CMV-IE promoter. Furthermore, the E3 region of Ad.TK^{RC} is removed and the E1b deletion is larger than in dl1520 (1257 *versus* 827 bp).

Since Ad.TK^{RC} carries all elements necessary for its replication in p53-deficient cells, both the original and subsequently infected p53-negative cells will be converted into adenoviral producer cells, promoting spread of the vector and thus the HSV*tk* suicide gene within tumors. In addition, the use of a single construct carrying the transgene and capable of replication is much less likely to result in the generation wild-type virus by homologous recombination than *trans*-complementation strategies using plasmid DNA or helper viruses. To reduce the likelihood of generating deletion mutants lacking HSV*tk* and yet expressing E1a and as a 'fail-safe' mechanism to allow us to abort the adenoviral infection with GCV treatment, we transcriptionally linked its expression to the HSV*tk* gene via an encephalomyocarditis virus internal ribosome entry site.[18]

Our studies comparing the *in vivo* efficacy of Ad.TK^{RC} *versus* a traditional replication-deficient Ad.TK in an A375 melanoma and ME180 cervical cancer xenograft model demonstrated three main findings. Antitumor responses induced by viral replication with Ad.TK^{RC} alone were equivalent to those achieved with Ad.TK plus GCV, confirming that the replication-competent adenovirus has direct oncolytic activity.[9,12] Secondly, there was no significant difference in survival among groups treated with Ad.TK combined with GCV, Ad.TK^{RC} alone or Ad.TK^{RC} followed by GCV treatment starting 1 day after vector administration. There was no difference in treatment efficacy, despite higher HSV*tk* expression in Ad.TK^{RC}-infected A375 cells (Figure 2), the anti-neoplastic activity of the E1a genes and their ability to act as a chemosensitizer by driving quiescent cells into S phase.[19] The higher expression of the HSV*tk* gene is likely due to vector replication and its preceding by the Ad5 TPL.[20,21] This suggests that the level of HSV*tk* expression as previously reported[22] and the presence of E1a, independent of its role in adenoviral replication, are not responsible for the significantly enhanced antitumor effect, compared with either treatment used alone, when GCV was given to Ad.TK^{RC}-treated mice after allowing time for viral replication. This observation demonstrates the importance of the amplification and spread of the viral inoculum beyond the initially infected cells by *in situ* conversion of the originally transduced cells into adenoviral producer cells.

The development of an immune response to these replicating adenoviral vectors will eventually limit their spread but should also impede re-treatment with the same or closely related adenovirus serotypes. However, in an immune-competent animal, treatment efficacy might also be enhanced by the augmented xenogenization of nontransduced tumor cells[23] due to increased transgene expression or presentation of the highly immunogenic E1a gene products in association with native tumor-specific antigens.[24,25] In addition, the expression of the E1a gene increases the susceptibility of cells to lysis by natural killer lymphocytes and macrophages.[26-28]

Bonnekoh and colleagues[29] also demonstrated that treatment of A375 melanoma xenografts with a replication-deficient adenovirus expressing HSV*tk* and GCV resulted in reduced tumor growth rates, but no complete responses were seen. The potentiation of the Ad.TK antitumor effect by inclusion of viral replication resulted in curative antitumor responses in 30–60% of treated animals and overall in a significant survival advantage in both the melanoma and cervical cancer model (39.5 *versus* 90+ and 64 *versus* 158+ days, respectively) thus representing a potential significant advance in vector design for cancer gene therapy.

Materials and methods

Generation of adenoviral vectors

Viral constructs were based on the plasmid system pBHG10, pBHG11 and pΔE1sp A (Microbix Biosystems,

Ontario, Canada).[30] Ad.TK^{RC} was generated by homologous recombination in 293 cells between pOW3–30, a pBHG11 derivative which lacks all Ad5 E1 sequences upstream of the pIX promoter, and the shuttle plasmid pAd.CMV-TKE1Δ55, which carries the HSV*tk*, E1a and E1b 19 kDa genes driven by the h-CMV-IE promoter in combination with the Ad5 tripartite leader (consisting of Ad5 DNA sequences from bp 6049 to 6288, 6795 to 7180, and 9644 to 9733),[31] as previously described.[32] In addition, we generated by standard methods[33] the replication-deficient adenoviruses Ad.GFP and Ad.TK, which carry the green fluorescent protein or the HSV*tk*, respectively, driven by the CMV–IE promoter.

Tissue culture

All cell lines were purchased from the American Type Culture Collection, Rockville, MD, USA and propagated in DMEM (GIBCO BRL, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (Hyclone, Logan, UT, USA) at 37°C and 5% CO_2.

Animal experiments

Female athymic nu/nu mice were obtained from Harlan-Sprague-Dawley (Indianapolis, IN, USA). Ten million A375 human melanoma cells or ME180 cervical cancer cells were injected subcutaneously into the right dorsal lumbar region in 100 µl serum-free DMEM with 10% Matrigel (Collaborative Products, Bedford, MA, USA). Both cell lines chosen are known to be p53 negative.[30,34] Blinded, bi-dimensional tumor measurements were performed twice a week with calipers, and tumor volume was determined using the simplified formula of a rotational ellipsoid ($l \times w^2 \times 0.5$).[35] Once tumors reached a volume of 100–150 mm^3 animals were treated with intratumoral injections of 10^9 adenovirus p.f.u. suspended in 100 µl of PBS or 100 µl PBS alone as a control. GCV 100 mg/kg (Roche Laboratories, Nutley, NJ, USA) was intraperitoneally administered in 1 ml of HBSS twice daily for 7 days beginning 1 day after virus inoculation, the optimal timing for the replication-defective Ad.TK, or after 3 days when the intratumoral Ad.TK^{RC} replication reached its maximum (data not shown).

Analytical and statistical methods

Survival analysis using the Cox–Mantel test and cumulative survival plots (Kaplan–Meier) were performed with STATISTICA release 5.1 for Windows (StatSoft, Tulsa, OK, USA).

HSVtk bystander assay

In vitro and *in vivo* bystander effect was assessed by using mixtures containing different ratios of Ad.TK or retrovirally STK[36]-transduced and untransduced cells, respectively, as previously described.[37]

Flow cytometric analysis of GFP expression

To determine the *in vitro* transduction efficiency with Ad.GFP, subconfluent cell-monolayers were exposed to a MOI of 10. Sixty hours later, single-cell suspensions were analyzed by flow cytometry. *In vivo* transduction efficiency was quantified by direct intratumoral injection of Ad.GFP into A375 xenografts. Sixty hours after virus injection the tumors were resected and enzymatically digested with 0.1% collagenase, 0.002% deoxyribonuclease and 0.01% hyaluronidase and subsequently analyzed by flow cytometry.

RNA dot-blot and Northern blot analysis

Twenty million A375 cells were exposed to the different adenoviruses at MOI of 25. Thirty-six hours later, polyadenylated RNA was isolated from the cells and RNA dot-blots were performed as previously described.[38] HSV*tk* expression of the various adenoviruses was assessed by Northern blot analysis following standard procedures.[39]

The blots were hybridized with the following α-^{32}P-dCTP-radiolabeled[40] probes: Ad5 E1a 12s (bp 560–893), Ad5 E1b 55 kD (bp 2400–3043), Ad5 L5 (bp 31043–31557) and the *BglII–NcoI* fragment of HSV-1 thymidine kinase gene.

Determination of GCV phosphorylation and incorporation into DNA

As previously described, the ability of Ad.TK^{RC} and Ad.TK-infected cells to phosphorylate GCV were analyzed by ion-exchange gradient elution HPLC[41] and the incorporation of tritiated GCV into genomic DNA by scintillation counting.[41,42]

Acknowledgements

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